Circadian Dynamics of Tumor Necrosis Factor α (Cachectin) Lethality

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Summary

Recombinant human tumor necrosis factor-α (TNF-α) has demonstrable antitumor activity in transplantable murine tumor models and patients with cancer but is highly toxic to both animals and human beings. The narrow therapeutic index of TNF-α has limited its anticancer utility. Toxicity associated with many standard anticancer drugs is highly dependent upon the circadian timing of their administration. The effect of time of day of TNF-α administration on lethal toxicity was examined in 238 BALB/c female mice in two studies. Each mouse received a single intravenous injection of human TNF-α at one of six equispaced times within the first contiguous 24-h cycle. The probability of dying across all times of day of TNF-α treatment was not equal ($p < 0.01$) and varied up to ninefold. Significant time of day dependence of TNF-α toxicity was present over a full order of magnitude of TNF-α dose. The frequency of TNF-α–induced lethality was greatest and the time to death was most brief when TNF-α was administered just before awakening. The survival probability was highest when TNF-α was administered in the second half of the daily activity span corresponding roughly to late afternoon and evening hours for human beings. The optimization of TNF-α administration timing is a strategy that warrants further investigation for improving the toxic/therapeutic ratio of this important cytokine. From a more fundamental perspective, these data may be essential for achieving a fuller understanding of TNF-α in vivo biology.

TNF-α has emerged as an important regulator of inflammation being one of the mediators of acute phase reactions, endotoxic shock, cachexia of chronic disease states, and cell-mediated host defense against bacteria, parasites, and tumor cells (1, 2). TNF-α induces hemorrhagic necrosis and regression of a variety of transplantable murine and human tumors (3–5). The mechanisms responsible for the antitumor action of TNF-α include both direct toxic effects upon tumor cells (6), indirect host-mediated procoagulant (7), and immunomodulatory effects which require host immune competency (4).

Therapeutically, recombinant human TNF-α has been investigated as a potential anticancer agent over the last decade for the treatment of patients with advanced cancer. Phase I clinical studies in cancer patients have, however, been daunting because of the severity of TNF-α toxicity (8–10). The endotoxin-like toxicities uniformly include hemodynamic instability, fever, diarrhea, metabolic acidosis, capillary leak syndrome, induction of a catabolic state, neurotoxicity, and renal and hematologic toxocity which severely limit drug dosing. At the same time, responses have been very infrequent with only minimal or partial responses observed.

These experimental and clinical data demonstrate that TNF-α can act either as a therapeutic tool or as a lethal toxic agent (11, 12). The narrow therapeutic index of TNF-α has encouraged the development of strategies to diminish toxicity while hopefully preserving antitumor activity. Several approaches include use of continuous infusions (13), glucocorticoids (14), inhibitors of prostaglandin synthesis (15), scavengers of oxygen radicals (16), and combining TNF-α with cytotoxic chemotherapy (17) or other cytokines (18). Toxicity still remains the most significant limitation to TNF-α therapeutic utility.

The toxicity of many standard cytotoxins in animals and humans can be reduced and dose intensity increased by appropriate circadian timing (19). In animal studies, optimal circadian drug timing has consistently been associated with maintenance of antitumor activity and has often improved tumor responses (20). In view of these findings and the realization that few, if any, biological processes do not participate in high-amplitude circadian rhythms, we examined the effect of circadian timing of TNF-α administration on lethal toxicity in BALB/c mice.

Materials and Methods

Animals and Chronobiologic Study Design. Female BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN), 8–10- (Study 1)

and 15–16-wk-old (Study 2), were studied. Mice were housed four to five per cage in three sound-dampened rooms under 12-h on, 12-h off light/dark schedules. The timing of these lighting regimens was staggered by 8 h in each of three different rooms. Time is referenced to as hours after light onset (HALO)[1] with lights on during the 0–12 HALO putative sleep span and lights off during the 12–24 HALO putative activity span of these nocturnal animals. Food and deionized water were freely available. Temperature and humidity were nearly constant at 24 ± 1°C and 40 ± 2%, respectively. Very low lux overhead red lights were used during the dark span to minimize animal disturbance and to facilitate human movement. Mice were standardized under these conditions for 3 wk before study. Circadian synchrony was documented by demonstration of the characteristic 24-h pattern in core body temperature before each experiment.

Cytokine Treatment. Recombinant human TNF-α was provided by Cetus Corp. (Emeryville, CA) as a lyophilized powder, and was >95% pure before lyophilization. Vials were reconstituted in PBS, pH 7.4, and stored −70°C before intravenous injection. Lot #TD 4 (1.3×10^7 U/mg sp act by the murine L929 assay) was used in Study 1. Lot #NP 102 (1.8×10^7 U/mg sp act) was used in Study 2. Animals were given a single intravenous bolus injection of TNF-α in 0.1–0.15 cc by tail vein at various doses (Million Units (MU)/kg body weight). Injections were performed at one of six equispaced circadian times (02, 06, 10, 14, 18, and 22 HALO) within the initial 24-h cycle.

Toxicity Study Design. Two large studies each involving the investigation of six groups of female BALB/c mice treated at one of six times spaced by 4-h intervals during the initial 24-h span were performed. In Study 1, a total of 119 mice were studied. Each of the six circadian treatment groups were randomly divided into four subgroups of five animals each (except for the 06 HALO group with 10 MU/kg, 4 mice were injected). Each subgroup received one of four randomly assigned doses of TNF-α (3.3, 6.7, 10.0, or 13.3 MU/kg). In Study 2, a total of 119 mice were studied. Each of the six circadian treatment groups was randomly divided into two subgroups of 10 animals each (except for the 02 HALO group with 27.0 MU/kg, nine mice were injected). Each subgroup received one of two doses of TNF-α (18.0 or 27.0 MU/kg). The final number of animal deaths per group (percent mortality) and the time to death (hours) after TNF-α injection of each animal (survival time) was recorded. Toxicity was assessed by inspecting cages every 2 h for animal deaths for up to 4 d. Proportional survival analyses were performed with data truncated at the time of last toxic death (98 h for Study 1 and 62 h for Study 2).

Statistical Analysis. For each study, the proportion of animals dead in each subgroup was compared by chi squared (χ^2) analysis. The percent survivors as a function of either dose or treatment timing was also evaluated by Kruskall-Wallis (KW) life table analysis. Analyses of variance (ANOVA, F value) were employed to determined the effects of TNF-α dose and circadian time of TNF-α injection upon survival time. The presence of circadian (sinusoidal) rhythm was assessed for the proportion of the treatment groups surviving and for the survival times by the cosinor least squares methods. (21).

LD_{50}. The lethal dose for 50% mortality (LD_{50}, dose of TNF-α that kills 50% of animals) is calculated by treating groups of animals with different doses across a range of doses that kills from a small minority of the exposed animals to well over half of them. Equal groups of the 238 mice in our study were treated with one of six TNF doses covering an order of magnitude of TNF-α (from ~3 to ~30 MU/kg) and at each of six equispaced times of day. To determine the relationship between dose and lethality, the mortality achieved at each dose was plotted against that dose and a line was fit by the process of least squares. This process was repeated for all dose groups treated at each of the six times of day yielding six separate lethality relationships with correlation coefficients (R) and six LD_{50} estimates. Linear regression of data grouped from treatments during sleep phase versus treatment during activity phase were also performed for calculation of LD_{50} of these grouped data.

[1] *Abbreviations used in this paper:* ANOVA, analysis of variance; HALO, hours after light onset; KW, Kruskall-Wallis; MU, Million units.

Results

Toxicity Studies

Survival Time Analysis. Time to death (survival time) truncated at the moment of last toxic death in each study was analyzed for dose and time of day effects. The mean survival times of these mice depend both upon the time of day of TNF-α administration, and upon the dose of TNF-α administered. As expected, the greater the TNF-α dose the shorter the survival time. A two-way ANOVA of the truncated survival time of mice treated with different TNF-α doses from each study separately and from both studies combined demonstrates a significant circadian time effect (Fig. 1 *A*, $F = 7.1$, $p = 0.0001$) as well as significant TNF-α dose–effect (Fig. 1 *B*, $F = 51.4$, $p = 0.0001$). An interaction between these two factors was also prominent ($F = 2.5$, $p < 0.001$), demonstrating that dose and timing have complex relations to mortality; e.g., lower doses at some times of day have greater lethality than much higher doses at other times of day. The mean truncated survival time of mice injected during the sleep span (02, 06, and 10 HALO) is much lower (43 h versus 68 h) than when the agent is given during the activity span (14, 18, and 22 HALO). The toxicity of TNF-α is greatest at 06 and 10 HALO (sleep phase) as evidenced by shorter survival times than at 14 and 18 HALO (late activity phase). Regardless of dose, time of day of TNF-α treatment was responsible for a 1.5–2-fold variation in survival times. The optimal times would approximate late afternoon and early evening for a diurnally active human being.

Analysis of Final Proportional Survival. Percent overall mortality as a function of the time of day mice received TNF-α and the dose of TNF-α was analyzed by χ^2 analyses for each study (Table 1). In Study 1, overall mortality ranged from 10 to 38% for all TNF-α doses. A significant circadian treatment time effect ($\chi^2 = 15.8$, $p = 0.008$) was demonstrated for the frequency of final overall mortality across all doses of TNF-α. The most statistically significant circadian time-dependent toxicity ($\chi^2 = 19.0$, $p = 0.002$) was seen at 10.0 MU/kg dose of TNF-α, where 100% of the mice died when treated just before daily awakening (10 HALO). This is contrasted to a total lack of mortality when mice receive the same dose of TNF-α during the middle of the activity span (18 HALO). Across all doses, mice treated during the second half of their daily activity phase (18 HALO) died less frequently (10% mortality), whereas mice treated just before awakening (10 HALO) showed a much higher final lethality (55% mor-

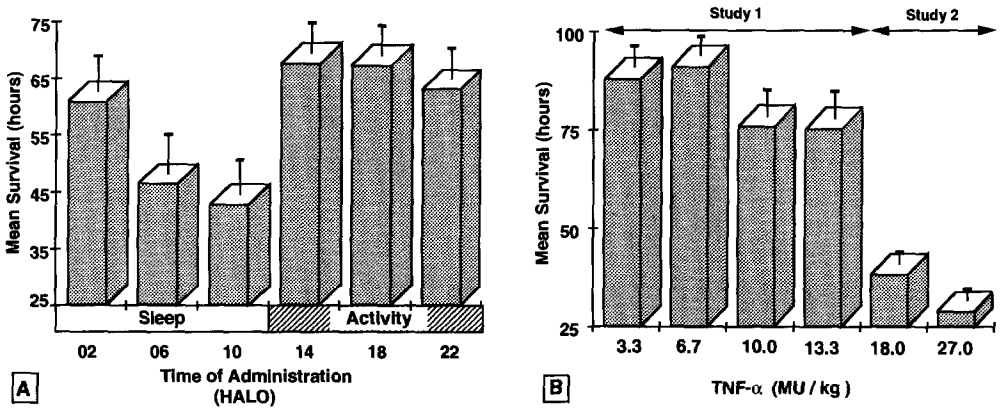

Figure 1. Observed survival time in hours (mean ± SEM) after TNF-α administration truncated at the time of last toxic animal death. (*A*) Survival time, across all TNF-α doses, of mice (*Study 1* and *Study 2* combined) as a function of the time of day (HALO) TNF-α was administered is shown. A significant time of day of treatment effect is seen across one entire log of TNF-α dose by ANOVA ($F = 7.1$, $p < 0.0001$). Survival time varied nearly twofold whether these studies were analyzed separately or together as shown here. The shortest survival time is seen when TNF-α is given just before daily awakening (10 HALO). (*B*) Survival time, across all times of day of TNF-α administration, of mice as a function of dose of TNF-α. A significant dose–effect is seen by ANOVA ($F = 51.4$, $p < 0.0001$) with higher doses resulting in shorter survival times.

Table 1. *Percent Mortality as a Function of the Time of Day of Administration (HALO) and Dose of TNF-α*

	Dose (MU/kg)	Time of Administration (HALO) 2	6	10	14	18	22	Mortality (All HALO)	χ^2 analysis χ^2	p value
				Truncated at time of last death						
Study 1	3.3	20 (1/5)	0 (0/5)	0 (0/5)	0 (1/5)	20 (2/5)	40* (2/5)	13.3 (4/30)	5.8	0.330
	6.7	0 (0/5)	20 (1/5)	40 (2/5)	0 (0/5)	0 (0/5)	0 (0/5)	10.0 (3/30)	7.8	0.170
	10.0	0 (0/5)	25 (1/4)	100 (5/5)	80 (4/5)	0 (0/5)	20 (1/5)	38.0 (11/29)	19.0	0.002
	13.3	20 (1/5)	40 (2/5)	80 (4/5)	0 (0/5)	20 (1/5)	20 (1/5)	30.0 (9/30)	9.0	0.110
	All doses	10 (2/20)	21.1 (4/19)	55 (11/20)	20 (4/20)	10 (2/20)	20 (4/20)	22.7 (27/119)	15.8	0.008
				Truncated at time of last death						
Study 2	18.0	90 (9/10)	100 (10/10)	50 (5/10)	30 (3/10)	60 (6/10)	40 (4/10)	61.7 (37/60)	16.4	0.006
	27.0	78 (7/9)	100 (10/10)	100 (10/10)	60 (6/10)	80 (8/10)	60 (6/10)	79.7 (47/59)	9.9	0.078
	All doses	84.2 (16/19)	100 (20/20)	75 (15/20)	45 (9/20)	70 (14/20)	50 (10/20)	70.6 (84/119)	20.6	0.001
				Truncated at 50% mortality						
Study 2	18.0	70 (7/10)	90 (9/10)	50 (5/10)	30 (3/10)	40 (4/10)	20 (2/10)	50.0 (30/60)	13.6	0.018
	27.0	56 (5/9)	100 (10/10)	80 (8/10)	30 (3/10)	20 (2/10)	30 (3/10)	53.0 (31/59)	20.4	0.001
	All doses	62.8 (12/19)	95.0 (19/20)	65 (13/20)	30 (6/20)	30 (6/20)	25 (5/20)	51.3 (61/119)	34.7	0.0001

* Percentage of mice dying after each dose of TNF-α administered at the referenced time of day in each study with number of dead mice/total mice shown in parentheses.

tality). In Study 2, overall mortality reached 62–80%. A significant circadian time-dependent effect (χ^2 = 20.6, p = 0.001) was also demonstrated for percent overall mortality of mice (Table 1). Mice treated during the activity phase (14, 18, and 22 HALO) showed lower mortality, whereas mice treated during the sleep span (02, 06, and 10 HALO) showed much higher mortality.

Life Table Analysis of Percent Survivors Over Time. KW life table analysis of percent survivors over time was analyzed for Study 2. A significant difference in overall survival pattern was seen with TNF-α dependent upon the time of day its administration for both the 18.0 MU/kg dose (KW score = 19.7, p <0.002; Fig. 2 *A*) and the 27.0 MU/kg dose (KW score = 17.4, p <0.05, Fig. 2 *B*). At both doses, mice treated in the middle and second half of the daily sleep span (06–10 HALO) died earlier and more frequently than those mice treated in the activity span (14–22 HALO).

LD_{50} Analysis and Mortality Truncated at 50%. The lethal dose for 50% survival (LD_{50}) was calculated from the results of all doses by linear regression of the dose–mortality relationship separately for each time of day of TNF treatment and for treatment in the light phase or in the dark phase. Results for LD_{50} estimates were 15.9, 13.5, and 12.9 MU/kg for treatment at 02, 06, and 10 HALO, respectively, and 14.1 MU/kg for treatment in the sleep phase (R = 0.79, p <0.001) compared with 24.0, 17.8, and 20.2 MU/kg for treatment at 14, 18, and 22 HALO, respectively, and 19.9 MU/kg for treatment in the activity phase (R = 0.83, p <0.001). Mice treated late in their activity phase tolerated much higher doses than mice treated just before early awakening. There was almost a twofold difference in LD_{50} (13 MU/kg vs. 24 MU/kg), depending solely upon the time of day that this cytokine was administered.

Proportional survival truncated when 50% of animals have died is an alternative way to look at time-dependent mortality. Since >50% mortality was achieved only in the Study 2, mortality truncated at 50% overall mortality was analyzed for this study (Table 1). Mice injected during the activity span (14–22 HALO) showed two to threefold lower mortality than those injected during the sleep span (02–10 HALO) assessed by percent mortality. The highest mortality of TNF-α was observed when administered at 06 HALO (95%) and the lowest mortality was seen when TNF-α was administered at 22 HALO (25%). The pattern and magnitude of these results are in accord with the LD_{50} calculations derived above.

Circadian Rhythm Analysis

Cosinor least squares analysis of a 24-h period fit was performed on the truncated survival time of TNF-α–treated mice (Table 2). In Study 1, a significant 24-h rhythm in TNF-α toxicity was found when all doses were examined together (p = 0.052). Across all doses, the time of day of treatment predicted for maximal survival (acrophase) is at end of the activity span (21:19 HALO). This same time of day was consistently found for each of the larger TNF-α doses. The lowest TNF-α dose (3.3 MU/kg), which killed only a tenth of the mice, predicted a somewhat different time of day for maximum survival time in the late sleep span (10:00). For Study 2, where overall mortality consistently exceeded 50%, a significant 24-h rhythm of TNF-α toxicity was also found (p <0.001). The predicted time of day for maximum survival (acrophase) was also found in mid-to-late activity (17:52 HALO). In both studies, the amplitude of the 24-h TNF-α toxicity rhythm was greater with higher doses of TNF-α, implying a greater proportion of the variability in toxicity was attributable to time of day-dependent differences. These analyses indicate that the safest time for TNF-α may be mid-to-late in the daily activity span, a time comparable to 4–10 p.m. for diurnally active human beings.

Discussion

At the end of last century, the medical community began paying attention to the therapeutic approach of Coley (22) who treated malignant tumors with repeated injections of

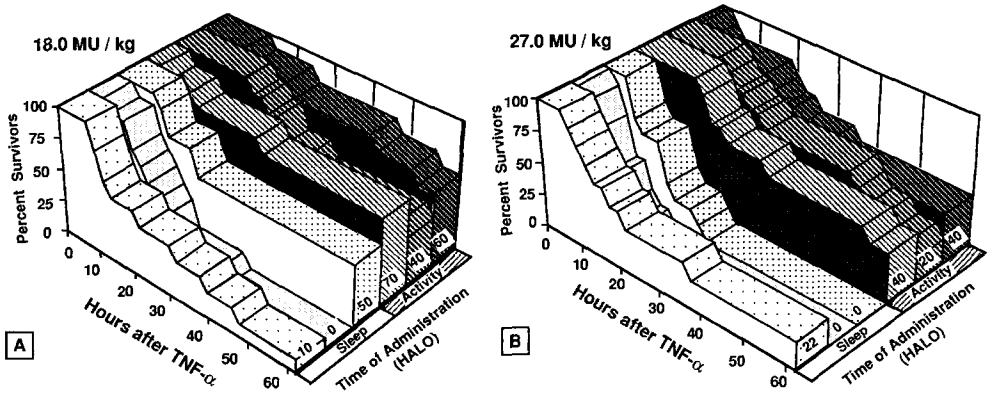

Figure 2. Life table analysis of percent survivors over time after TNF-α is demonstrated as a function of time of day of administration (Study 2). TNF-α was administered at one of six times within the first 24-h cycle at a dose of 18.0 (*A*) or 27.0 MU/kg (*B*). Kruskall-Wallis analyses reveal a significant difference in the proportion of survivors dependent upon when within the day TNF-α is administered for both the 18.0 MU/kg dose (KW value = 19.7, p <0.002) and the 27.0 MU/kg dose (KW value = 17.4, p <0.05). Mice given TNF-α during the sleep span (02, 06, 10 HALO) have threefold higher survival rates than mice given TNF-α in the activity span (14, 18, 22 HALO). The number at the base of each survival curve is the final percentage of survivors truncated at 62 h (time of last death) for each HALO of treatment.

Table 2. *Cosinor Analysis of the Time of Day (HALO) Dependence of Survival Time (h) of Mice after TNF-α Administration*

	Dose (MU/kg)	No. of Mice	*p* Value	Mesor	Amplitude	Acrophase (HALO)
				h		
Study 1	3.3	30	0.073	87.4 ± 4.9*	16.8 ± 7.0‡	10:00 ± 1:36§
	6.7	30	0.086	90.5 ± 4.3	14.1 ± 6.1	20:30 ± 1.42
	10.0	29	0.012	75.1 ± 5.9	26.7 ± 8.2	22.42 ± 1:12
	13.3	30	0.130	74.7 ± 6.7	19.8 ± 9.4	20.33 ± 1:48
	All doses	119	0.052	82.0 ± 3.0	10.3 ± 4.2	21:19 ± 1.53
Study 2	18.0	60	<0.001	38.2 ± 2.5	15.3 ± 3.5	17:16 ± 0:54
	27.0	59	<0.001	28.6 ± 2.3	17.0 ± 3.2	18:23 ± 0.42
	All doses	119	<0.001	33.5 ± 1.7	16.0 ± 2.5	17:52 ± 0:36

Cosinor analysis was performed with a 24-h period fit.
* Mesor, 24-h adjusted mean for the survival time (h).
‡ Amplitude, half the difference between maximal and minimal values for survival time.
§ Acrophase, time of day expressed in HALO for the maximum predicted survival time that was most consistently between 17:00 and 22:00 HALO (mid to late sleep). Values are given with the standard error.

bacterial cultures. Later, the immune and inflammatory reactions, known as the Shwartzman phenomenon, associated with bacterial toxin administration, were observed to be associated with local and sometimes generalizable anticancer effects (23, 24). A tenacious search for the factors responsible for the tumor hemorrhage associated with the administration of these toxins concluded with the identification and isolation of endotoxins. Associated with these toxin exposures was the release of a series of powerful cytokines. TNF-α was finally identified as a prominent component of this inflammatory process (25, 26). It was found that TNF-α could reproducibly cause shrinkage of and even cure transplantable, chemically induced cancers (meth-A sarcoma) in BALB/c mice (27). The therapeutic responses in animals and humans, however, have usually been achieved at doses of TNF-α approaching those associated with endotoxin-induced shock (2, 11, 12).

We have found that the timing within the day of TNF-α administration to BALB/c mice is responsible for up to a ninefold difference in the frequency of lethal toxicity and time to death over a range of TNF-α doses. As the dose of TNF-α increased, the time of day difference in this predictable toxicity rhythm becomes greater. In light of the central importance of TNF-α in mediating endotoxin-induced lethality, it is interesting to note a circadian-timed endotoxin study done more than 30 yr ago. Halberg et al. (28) reported that the susceptibility of mice to *Escherichia coli* endotoxin–induced lethality varied significantly and predictably throughout the day. When endotoxin was administered during the activity span, a 10-fold-lower mortality of BALB/c mice reproducibly occurred. This finding accurately predicts and is coincident with the circadian toxicity pattern for TNF-α demonstrated here. In D-galactosamine–loaded mice (to enhance endotoxin susceptibility), a ninefold difference in the susceptibility to endotoxin or monophosphoryl lipid A–induced lethal toxicity was seen with greatest toxicity when the agents were administered at the sleep–wake border (29). The diurnal variation in endotoxin toxicity reported with D-galactosamine–loaded mice was paralleled by circadian-dependent elevations in serum TNF-α levels.

TNF-α is clearly a final common pathway for the induction of endotoxin-associated shock secondary to sepsis. It is interesting to note that hospital deaths, many associated with sepsis, do not occur randomly throughout the day but are more frequent between 2 a.m. and 6 a.m. (30). This time of day corresponds to the time in the circadian cycle when endotoxin and TNF-α are each most frequently lethal. The recognition and explanation of this time structure may well be helpful to the successful use of the many potential molecular blockers of sepsis-induced lethality that are currently under aggressive clinical study.

The underlying mechanisms for this time of day toxicity are not known. The peak toxicity for TNF-α and *E. coli* endotoxin both occur at the time of day that is close to the usual daily cortisol peak (31). Very large and reproducible circadian-dependent variations in the production and secretion of glucocorticoids, adrenocorticotropic hormone and growth hormone have been documented. These same hormones are also important in mediating the response to acute infectious and endotoxic challenge. Pharmacological manipulation of glucocorticoid levels can influence the response to TNF-α and endotoxin. Treatment of cells in vitro with high doses of glucocorticoid can modulate the production of several inflammatory cytokines including TNF-α (32), IL1 (33), IFN-γ (34), and IL-2 (35). Adrenalectomy has been reported to sensitize mice to the lethal response to endotoxin (36, 37). Endotoxin causes increased glucocorticoid production and mice rendered tolerant to TNF in vivo have increased glucocorticoid production. However, in normal animals, glucocorti-

coid receptor antagonists did not prevent endotoxin effects (38). How well these extreme manipulations of glucocorticoids relate to the endogenous circadian change in glucocorticoid levels is not clear. Hypophysectomy can increase the sensitivity to lethality of bacterial inoculation (39) and pituitary-hypophyseal–derived proteins appear to modulate this response. Growth hormone protects hypophysectomized mice from lethal bacterial challenge (39), whereas pituitary production of macrophage migration inhibitory factor (MIF) or administration of MIF enhances the lethality of endotoxin (40). Serum levels of IL-1 vary throughout the day (41) which could conceivably modulate TNF effects and cytokine cascades. Mechanisms by which the normal physiologic circadian variation in the production of these hormones and their response to physiologic modulation contributes to circadian-dependent lethality of TNF and endotoxin warrant investigation.

Other mechanisms may also contribute to circadian-dependent differences in TNF-α–induced lethality. Daily variations in the function of target tissues exist and could modulate the susceptibility of these tissues to TNF-α–mediated toxicity. For example, circadian-dependent variations in the glutathione cycle activity (20), in immune cell function (42), bone marrow function (43), and tumor blood flow (44), have all been described. Time of day-dependent differences in protein drug pharmacokinetics may also contribute to time of day toxicity profiles.

Circadian timing of drug administration, as a strategy to alter dose-limiting toxicities, has been demonstrated in preclinical and clinical studies with traditional agents. Circadian timing of antitumor or hematopoietic cytokines may be an additional strategy to alter dose-limiting toxicities and/or improve efficacy. The circadian time structure of therapeutic cytokine administration is beginning to be investigated. Myelotoxicity and antitumor activity of IFN-α and IFN-γ have been demonstrated to vary as a function of circadian time of administration in mice (45–47). The optimal time for minimal myelotoxicity and maximal antitumor activity were coincident for each type of IFN but with different optimal times of day for the two classes of IFNs. Hematopoietic growth factor efficacy also varies with the time of day of their administration (48). Our current studies suggest that TNF-α toxicity is highly dependent upon the time of day of administration. Optimal circadian timing of TNF-α administration might be a useful strategy to consider to increase dose intensity and possibly enhance antitumor efficacy.

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